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LED fluorescence spectroscopy for direct determination of monoamine oxidase B inactivation

J. Castillo [a,*], J. Hung [b], M. Rodriguez [a], E. Bastidas [a], I. Laboren [a], A. Jaimes [a]

[a] *Escuela de Química, Universidad Central de Venezuela, Los Chaguaramos, Caracas 1080A, Venezuela*
[b] *Centro de Física, Instituto Venezolano de Investigaciones Científicas, Caracas 1020A, Apartado 21827, Venezuela*

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Abstract

In this work, we report an alternative assay for the determination of the inhibitory effect on monoamine oxidase B (MAO-B) activity of probe compounds. Enzyme MAO-B exhibits fluorescence emissions when it is excited at 412 nm. Using an inexpensive blue LED-like excitation source, we measured the quenching of fluorescence intensity of MAO-B enzyme during the reaction with inhibitors. The applicability of the procedure is demonstrated by assays with L-deprenyl and berberine as inhibitors through the use of fluorescence studies. The IC_{50} values of L-deprenyl and berberine were 0.04 and 90 μM, respectively. The K_I values were 0.020 and 47 μM for L-deprenyl and berberine, respectively. These IC_{50} and K_I values were similar to the values obtained with a standard method. These results demonstrate the feasibility of this method as an alternative to follow the inhibitory effect on MAO-B.
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It is well known that monoamine oxidase (MAO)[1] (EC 1.4.3.4), an integral membrane-bound flavoenzyme situated on the mitochondrial outer membrane, plays an important physiological role in the regulation of the levels of neurotransmitter monoamines in various organs and plays a protective role by the inactivation of potentially toxic exogenous monoamines [1–3]. MAO exists as two isozymes, designated as MAO-A and MAO-B, that regulate the tissue levels of amine neurotransmitters and protect the animal from the effects of pharmacologically active dietary amines. MAO-A inhibitors are used clinically in the treatment of depression, whereas inhibition of MAO-B may alleviate symptoms and slow the progression of Parkinson's disease [4,5].

Currently, a variety of assay protocols exist to exanimate and quantify the inhibitory effects of drugs on MAO activity [6–8]. In general, these methods are based on the measurement of the conversion of an amine substrate to aldehyde [6]. Compounds such as benzylamine, phenylethylamine, kynuramine, and serotonine have been widely used as substrates, and the activity is followed by a measure of the variation in substrate concentrations through the absorption or fluorescence intensity variations [2,9–11] where the measure signal corresponds to the substrate molecule. This makes the standard a direct assay for conversion activity but makes it an indirect assay if the interest is to measure the inhibition.

Fluorometric analyses are at least 100- or even 1000-fold more sensitive than the absorption method [12]. In general, these methods are indirect and have been widely used due to their relative simplicity and high sensitivity. However, relatively high concentrations of purified enzyme are required, and the assays are limited to experiments with one substrate. In addition, assays that

* Corresponding author. Fax: +58 212 6052246.
E-mail address: jimmy@strix.ciens.ucv.ve (J. Castillo).
[1] *Abbreviations used:* MAO, monoamine oxidase; MAO-A, monoamine oxidase A; MAO-B, monoamine oxidase B.

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measure ammonia suffer from lack of sensitivity and are useful only for oxidation of primary amines [9,10].

The literature contains fluorescence spectroscopic studies of enzyme MAO-B. Enzyme MAO-B exhibits fluorescence emissions when it is excited at 412 nm [13,14]. There are well-known intrinsic fluorophores of the enzyme that fluoresce in the visible spectral region. The enzyme-bound flavin is mainly responsible for the fluorescence of enzyme MAO-B. Its emission spectra are significantly sensitive to the local environment as well as to specific association of the enzyme with substrates or other molecules as the inhibitors. MAO inhibitors have been studied for more than a half-century, and several aspects of the enzyme (e.g., binding site, chemical mechanisms) are still the focus of debate. All of the mechanisms proposed so far are in relation to the charge transfer mechanism. As a result, one may expect that the variations in fluorescence intensity of the enzyme are dependent on those factors that affect the MAO-B inhibitory action of drugs. Woo and Silverman [13] reported a decrease in the emission intensity of the flavin group of MAO-B by covalent binding of pargyline (enzyme inhibitor). More recently, researchers have demonstrated the application of fluorescence spectroscopy to the study of enzyme active sites [15,16].

In this sense, we proposed an alternative method to determine the activity of MAO-B efficiently and rapidly through variations in the intensity of fluorescence of MAO-B enzyme reaction with an inhibitor without the need for a substrate.

L-Deprenyl (phenyl-isopropyl-methyl-propargyl-amine) plays an essential role in MAO research and is considered the "gold standard" of MAO-B. A significant portion of the clinical data and biological actions of MAO-B inhibitors is based on the effects of deprenyl. It is known that deprenyl has a rather complex pharmacological effect on MAO-B activity and acts as a MAO-B inhibitor in a determined concentration range, inducing important effects in the enzyme [5,17]. Its characteristics of fluorescence are not found in the literature, but its important action on the enzyme can result in variations in the fluorescence intensity that will be dependent on concentration of the drug.

To demonstrate our alternative methods in the evaluation of inhibitor effect on MAO-B without the need for substrate, we determined the inhibition of MAO-B activity of the inhibitor L-deprenyl and the berberine hydrochloride, a protoberberine alkaloid with important inhibitory effects on MAO-B activity [7]. In this technique, the fluorescence source is an inexpensive super-brilliant LED with a maximum emission at 380 nm. The results obtained are compared with those using the traditional indirect method. Good agreement was obtained for the inhibitor (L-deprenyl) and the berberine alkaloid.

Materials and methods

Materials and preparation of MAO-B

Mouse liver mitochondrial MAO-B was isolated by the method of Lu and coworkers [18] with a slight modification. Activity of MAO-A declined rapidly, with a half-life time of approximately 24 h; therefore, all of our results are from enzyme MAO-B. MAO-B was purified by isolation of mitochondria from liver homogenates. Liver tissue (4 g) was homogenized 1:40 (w/v) in 0.25 M sucrose. Following centrifugation at 1000*g* for 10 min, the supernatant was further centrifuged at 10,000*g* for 30 min to obtain a crude mitochondrial pellet. The pellet was resuspended in 4 ml of 0.25 M sucrose and was layered onto 20 ml of 1.2 M sucrose. A mitochondrial pellet was obtained by centrifugation at 105,000*g* for 1 h. Following a single wash in potassium phosphate buffer (200 mM, pH 7.6), mitochondria were suspended in 16 ml of buffer and stored in 1-ml aliquots at −70 °C until use.

MAO-B activity was adjusted to 8.0 ± 0.5 nmol/h/mg protein for the assays. Proteins levels were determined by the modified method of Lowry and coworkers [19] using bovine serum albumin as a standard. Finally, the enzyme was diluted to one-eighth of the initial concentration for the studies with the samples.

Solutions ranging from 0.001 to 0.025 μM of deprenyl solution in potassium phosphate buffer (100 mM, pH 7.6), and solutions ranging from 0 to 100 μg/ml of berberine solution in Tris–hydrochloride acid buffer (20 mM, pH 9.1), were used. An incubation of inhibitor solutions with 12 μl of enzyme MAO-B was carried out.

Absorption and fluorescence measurements

Absorption spectra of all samples were measured at room temperature using a diode array spectrometer of 0.5 nm spectral resolution between 200 and 1000 nm (model 2200 UV–Vis, Ocean Optics). The absorption spectra of samples were corrected with the solvent spectra.

Fluorescence spectra were measured using a classic optic mount at 90° signal observation. For an excitation source, a light-emitting diode with an emission maximum at 398 nm and a half-width of 50 nm was used. The fluorescence spectra were recorded on a fiber-optic spectrometer (model 2200 UV–Vis).

Assay procedure

MAO-B activity was measured by the changes in fluorescence intensity of the enzyme after reaction with different concentrations of L-deprenyl and berberine

Table 1
IC_{50} and K_I values for L-deprenyl and berberine by benzylamine (substrate) method and direct fluorescence method

Compound	Benzylamine (substrate) method		Direct fluorescence method (without substrate)		Literature	
	IC_{50} (μM)	K_I (μM)	IC_{50} (μM)	K_I (μM)	IC_{50} (μM)	K_I (μM)
L-Deprenyl	0.03	0.018	(0.04 ± 0.01)	(0.020 ± 0.006)	0.03–0.06 (Ref. [20])	0.017 (Ref. [18])
Berberine	98.4	—	(90 ± 5)	(47 ± 4)	98.2 (Ref. [7])	44.2 (Ref. [7])

for 30 min at 37 °C using an LED diode at 398 nm as excitation wavelength. To compare our assays, we also carried out the study of inactivation of MAO-B with the standard method using benzylamine as substrate through the formation of benzaldehyde from benzylamine, followed by UV–Vis spectroscopy assay at 290 nm [16].

Results and discussion

Measurement of MAO-B activity using benzylamine as substrate

Using the method proposed by Lu and coworkers [18], we determined the IC_{50} values of L-deprenyl and berberine (Table 1). L-Deprenyl and berberine K_I values were also determined using the method of Kenzi and coworkers [16]. As can be observed, the IC_{50} values (concentrations given to 50% MAO-B inhibition) and the obtained K_I values of deprenyl and berberine are in agreement with the values reported by Lee and coworkers [7] and others [20,21].

Spectroscopic characterization of enzyme MAO-B, deprenyl, and berberine

Fig. 1 shows the absorption spectrum of MAO-B, displaying two absorption maxima: one at 412 nm and the other at 434 nm. The absorbances of these bands are due to the existence of both an oxidized flavin and a flavin semiquinone [13]. When enzyme MAO-B is excited at a wavelength of 398 nm, the fluorescence spectrum (Fig. 1 inset) exhibits a maximum emission at 502 nm that is characteristic of enzyme-bound flavins. This maximum depends strongly on the environment; the flavoprotein is known due to its fluorescence between 500 and 530 nm [12–14].

Fluorescence and absorption spectra of L-deprenyl solution are displayed in Fig. 2. As is illustrated in the figure inset, the L-deprenyl does not exhibit absorption in the visible region; however, when it is excited at a wavelength of 398 nm, it produces a small induced emission with a maximum at 520 nm. The maximum of fluorescence intensity shows a linear relationship at different concentrations for the deprenyl solution in potassium phosphate buffer (0.001–0.025 μM). In this range of concentrations, the intensity of the fluorescence spectra is at least one order of magnitude smaller than that for the enzyme.

The UV–Vis absorption spectrum and the structure of berberine are shown in Fig. 3. The spectrum displays three absorption maxima. The absorption at 262 nm and that at 344 nm correspond to the isoquinoleic structures that are characteristic of the transitions π to π*, and the band at 424 nm is due to the transitions $n - \pi^*$ characterized by a broad band and low in intensity. In addition, the fluorescence spectrum of berberine solution is shown in Fig. 3. This spectrum shows a broad band with a maximum emission at 560 nm when it is excited at 398 nm. A linear relationship of fluores-

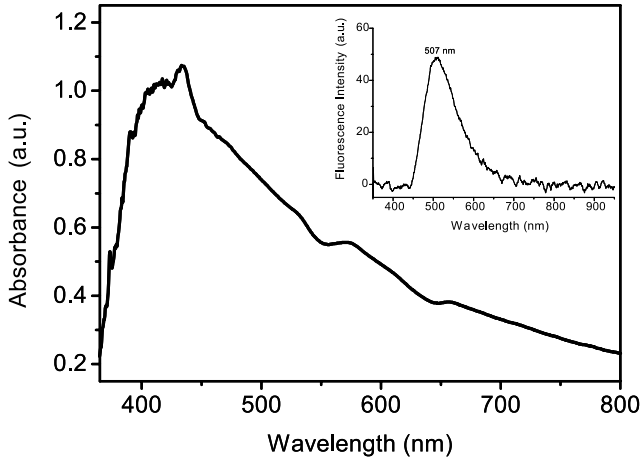

Fig. 1. Absorption and fluorescence (inset) spectra of MAO-B.

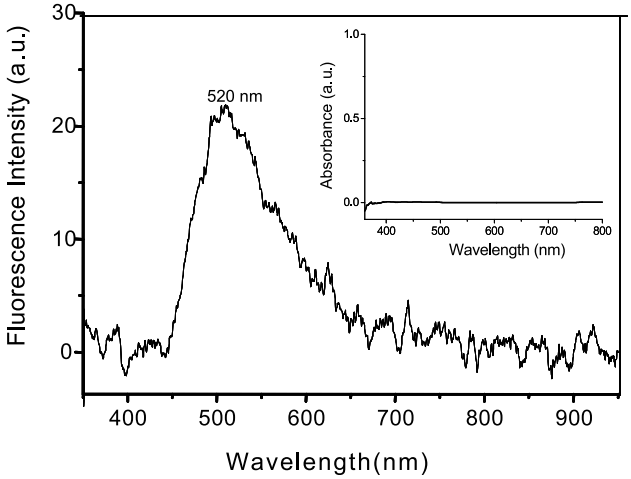

Fig. 2. Absorption and fluorescence (inset) spectra of L-deprenyl solution (0.04 μg/ml).

cence intensities at different concentrations studied was observed for the berberine solution in Tris–hydrochloride acid buffer (0–100 μg/ml) as well.

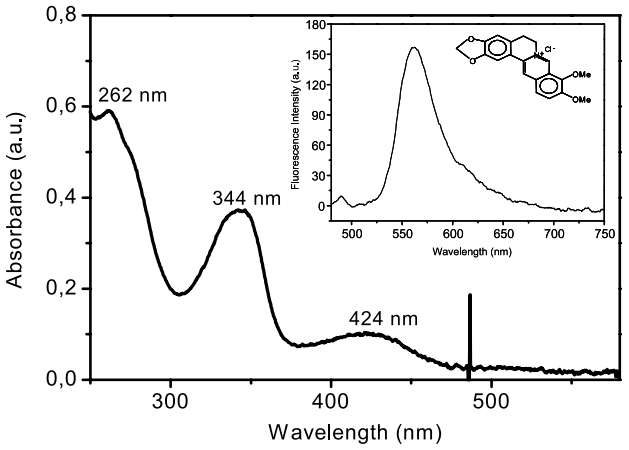

Fig. 3. UV–Vis absorption spectrum of berberine solution (1.38 mM). The inset shows fluorescence emission spectra of berberine solution (100 μg/ml).

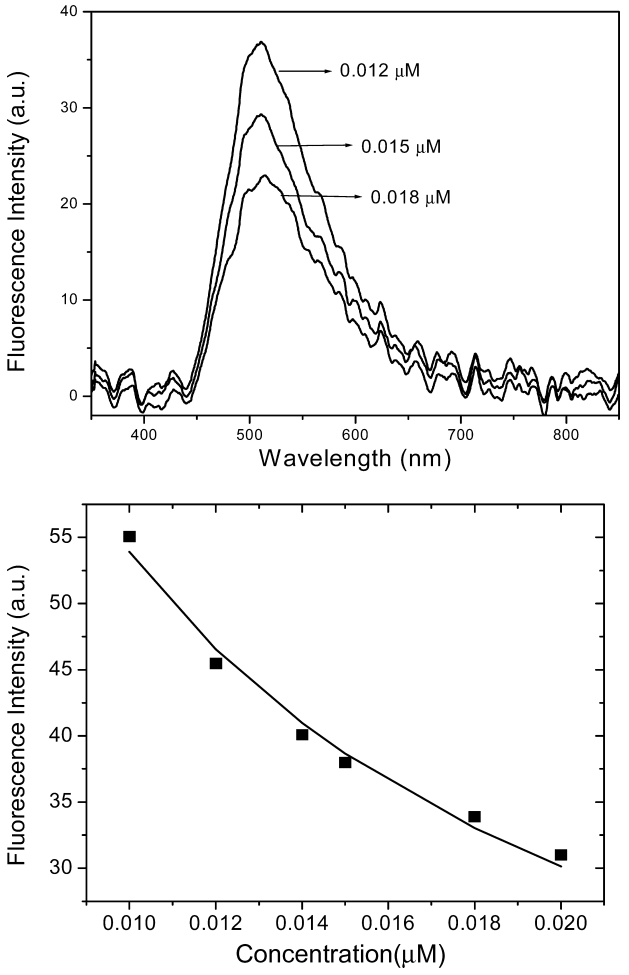

Fig. 4. Fluorescence spectra (upper panel) at different concentrations of L-deprenyl solution with the enzyme and maximum of fluorescence intensity (lower panel). The points correspond to experimental data, and the line corresponds to the fit using the inverse of Eq. (4).

Effect of L-deprenyl and berberine on MAO activity by LED fluorescence

The upper panel of Fig. 4 shows the fluorescence spectra for enzyme MAO-B at different concentrations of L-deprenyl added. The change in the maximum of fluorescence intensity appears to be due to the quenching effect on the enzyme resulting from the interaction of the L-deprenyl with the enzyme. This behavior is similar to that reported in enzyme-binding experiments where the quenching of the fluorescence intensity is due to complex formation [16]. This dependence in the maximum of fluorescence intensity at different concentrations is shown in the lower panel of Fig. 4. The first point in the plot corresponds to a free enzyme, and the rest of the points correspond to the quenching of fluorescence intensity from the enzyme bound to L-deprenyl. When the reaction is completed, the signal is suppressed at a minimum. For the L-deprenyl solution, the IC_{50} is 0.04 μM, which is in good agreement with the range of the reported and obtained values using other methods [21].

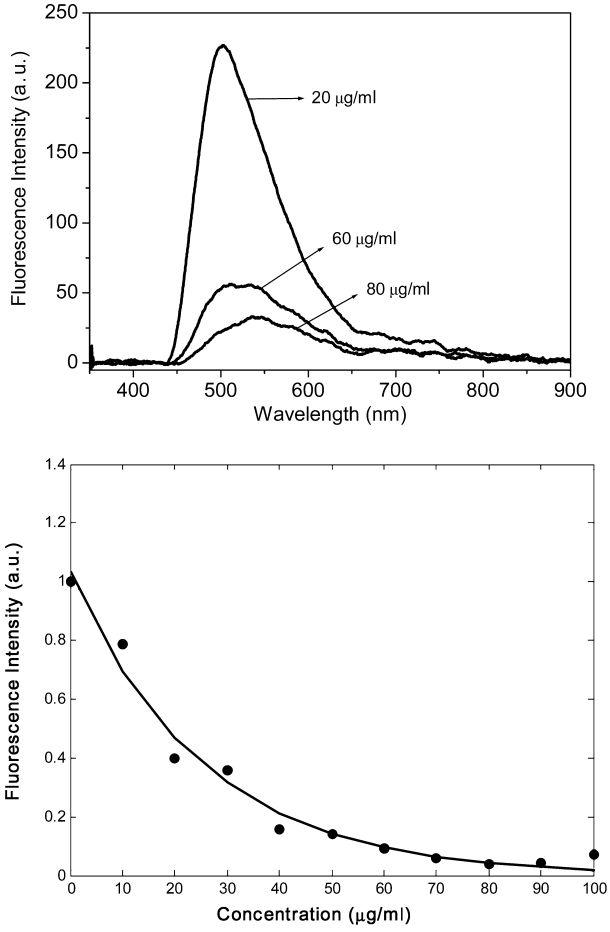

Fig. 5. Fluorescence spectra (upper panel) at different concentrations of berberine solution with the enzyme and maximum of fluorescence intensity (lower panel). The points correspond to experimental data, and the line corresponds to the fit using the inverse of Eq. (5).

The upper panel of Fig. 5 shows the fluorescence spectra for enzyme MAO-B with different concentrations of berberine added. The lower panel shows the variations in the maximum of fluorescence intensity at different concentrations of berberine. This behavior is a consequence of the quenching of fluorescence due to enzyme bound to berberine. For the berberine solution, the IC_{50} value obtained is 90 μM, which is also in good agreement with the values reported by Lee and coworkers [7].

The quenching effect of the compounds studied on MAO-B fluorescence can be explained through of the following inhibition mechanism of MAO-B. The enzyme [E] (fluorescent) reacts with the inhibitor [I] (nonfluorescent or very low fluorescent) to form an irreversible enzyme inhibitor [EI] (nonfluorescent complex), which reacts further to form the irreversible covalent enzyme adduct [E − I] [12]:

$$[\mathrm{E}] + [\mathrm{I}] \overset{K_i}{\rightleftarrows} [\mathrm{EI}] \rightarrow [\mathrm{E} - \mathrm{I}], \tag{1}$$

where $K_i = k_{-1}/k_1$. This is an apparent rate constant for the formation of nonfluorescent complex. The efficiency of the complex formation is proportional to the quenching of fluorescence. When the quencher is inefficient, the dependence of K_i with variables such as solvent effect, viscosity, and temperature may be complicated and the quenching of the fluorescence may disappear. This effect is already associated with the enzyme inactivation induced by the quencher. In the stationary state, we can consider that the dependence of the fluorescence intensity on quencher concentration is derived by consideration of the association constant for complex formation. This constant is given by

$$K_\mathrm{s} = \frac{[\mathrm{E} - \mathrm{I}]}{[\mathrm{E}][\mathrm{I}]}, \tag{2}$$

where [E − I] is the concentration of the enzyme–inhibitor complex and [E] is the concentration of uncomplexed fluorophore. If the complex species are nonfluorescent, the fraction of the fluorescence (E/E_0) is given by the fraction of the total fluorophores that is not complexed (f), with $f = F/F_0$. Recall that the total concentration of fluorophore $[E]_0$ is given by

$$[\mathrm{E}]_0 = [\mathrm{E}] + [\mathrm{E} - \mathrm{I}]. \tag{3}$$

We can substitute the fluorophore concentrations with the fluorescence intensities, and solving Eq. (3) yields

$$\frac{F_0}{F} = 1 + K_\mathrm{I}[\mathrm{I}]. \tag{4}$$

This is the classical Stern–Volmer equation, which relates the drop in fluorescence. Plot F_0/F versus [I] is a straight line with the slope equal to K_I, and this constant is often related to the complex formation. Fig. 6A shows the plot of F/F_0 versus [I] for the enzyme–L-deprenyl reaction, with the slope corresponding to the K_I value. This value of 0.020 μM obtained is in agreement with the values reported in the literature [18] and with those measured using the standard method (Table 1). For the enzyme–berberine reaction, the result is presented in the plot shown in Fig. 6B. In this case, a nonlinear response for F_0/F versus [I] is obtained. This variation can be explained by the existence of both dynamic and static quenching processes. In this case, the fit equation is as follows [12]:

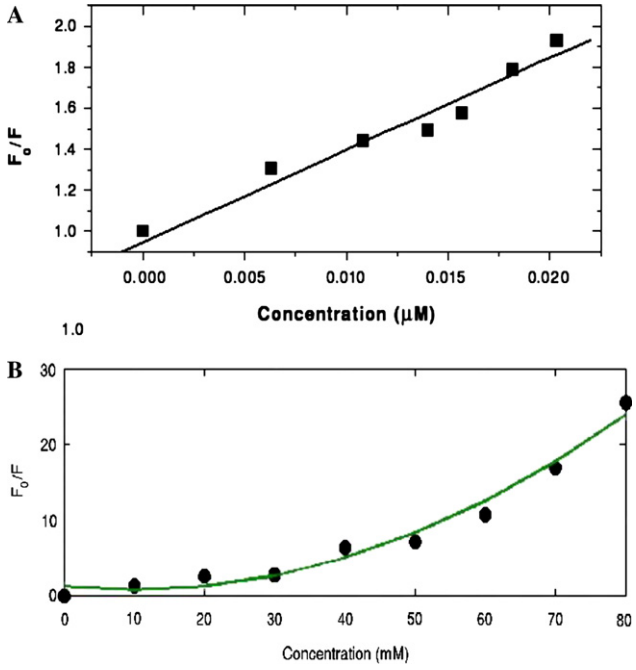

Fig. 6. Plot of F_0/F versus [I] for the enzyme–L-deprenyl reaction (A) and enzyme berberine reaction (B).

$$F_0/F = 1 + (K_\mathrm{I} + K_\mathrm{d})[\mathrm{I}] + K_\mathrm{I}K_\mathrm{d}[\mathrm{I}]^2, \tag{5}$$

where K_d is the collision quenching constant. Fitting the data with a second-order equation is possible to obtain the K_I constant, which was 47 μM. This value is in excellent agreement with the values reported in literature and with those measured using the standard method (Table 1).

Conclusions

The LED fluorescence method presents an alternative for evaluating the inhibitory effects on MAO-B activity without the use of any substrate. The assay is suitable for estimations of inhibitor potentiality through IC_{50} values and through determinations of kinetic constants. The IC_{50}

and K_I values of two inhibitors tested, L-deprenyl and berberine, were measured to probe the capability and applicability of the method. The values obtained are in good agreement with those reported using the standard method (i.e., using benzylamine as substrate). The results of this study demonstrate a useful and rapid alternative method for identifying potential inhibitors of MAO-B.

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